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Vasoactive intestinal peptide exerts an excitatory effect on hypothalamic kisspeptin neurons during estrogen negative feedback

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ABSTRACT

Hypothalamic kisspeptin neurons are the primary modulators of gonadotropin-releasing hormone (GnRH) neurons. It has been shown that circadian rhythms driven by the suprachiasmatic nucleus (SCN) contribute to GnRH secretion. Kisspeptin neurons are potential targets of SCN neurons due to reciprocal connections with the anteroventral periventricular and rostral periventricular nuclei (AVPV/PeN) and the arcuate nucleus of the hypothalamus (ARH). Vasoactive intestinal peptide (VIP), a notable SCN neurotransmitter, modulates GnRH secretion depending on serum estradiol levels, aging or time of the day. Considering that kisspeptin neurons may act as interneurons and mediate VIP's effects on the reproductive axis, we investigated the effects of VIP on hypothalamic kisspeptin neurons in female mice during estrogen negative feedback. Our findings indicate that VIP induces a TTX-independent depolarization of approximately 30% of AVPV/PeN kisspeptin neurons in gonad-intact (diestrus) and ovariectomized (OVX) mice. In the ARH, the percentage of kisspeptin neurons that were depolarized by VIP was even higher (approximately 90%). An intracerebroventricular infusion of VIP leds to an increased percentage of kisspeptin neurons expressing the phospho^{Ser133} cAMP-response-element-binding protein (pCREB) in the AVPV/PeN. On the other hand, pCREB expression in ARH kisspeptin neurons was similar between saline- and VIP-injected mice. Thus, VIP can recruit different signaling pathways to modulate AVPV/PeN or ARH kisspeptin neurons, resulting in distinct cellular responses. The expression of VIP receptors (VPACR) was upregulated in the AVPV/PeN, but not in the ARH, of OVX mice compared to mice on diestrus and estradiol-primed OVX mice. Our findings indicate that VIP directly influences distinct cellular aspects of the AVPV/PeN and ARH kisspeptin neurons during estrogen negative feedback, possibly to influence pulsatile LH secretion.

1. Introduction

The preovulatory peak of the luteinizing hormone (LH) occurring in the late afternoon of proestrus in rodents is synchronized by circadian cues (Bronson and Vom Saal 1979; Christian et al., 2005). The suprachiasmatic nucleus (SCN), which drives circadian rhythms, is comprised of a complex and heterogeneous population of cells that are responsible for the synthesis and release of various biogenic amines and neuropeptides, including the vasoactive intestinal peptide (VIP) (Moore and Speh 1993; Moore et al., 2002). SCN cells modulate the activity of gonadotropin-releasing hormone (GnRH) neurons by at least three main mechanisms. The first mechanism involves direct innervation of GnRH neurons by SCN terminals (de la Iglesia et al., 1995; Van Der Beek et al., 1997; van der Beek et al., 1993), and the second and third refer to reciprocal connections between the SCN and the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus of the hypothalamus (ARH) (Abrahamson and Moore 2001; Gu and Simerly 1997; Hahn and Coen 2006; Saeb-Parsy et al., 2000; Simerly and Swanson 1987; Van Der Beek et al., 1997; Vida et al., 2010; Watson et al., 1995; Wintermantel et al., 2006; Yi et al., 2006).

It is well known that the AVPV and the rostral periventricular nucleus (here referred to as AVPV/PeN) and the ARH are composed of various cell types, including *Kiss1*-expressing cells, known as kisspeptin neurons (Gottsch et al., 2004). Kisspeptin neurons coexpress the

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estrogen receptor alpha, among numerous other genes (Smith et al., 2005a; Smith et al., 2005b; Stephens and Kauffman 2021). *Kiss1* expression in the AVPV/PeN temporally increases in late afternoon of proestrus or in late subjective day in OVX estradiol (E2)-primed female mice (Poling et al., 2017; Robertson et al., 2009). AVPV/PeN kisspeptin neurons project to GnRH soma in the preoptic area and to distal terminals near the median eminence, exhibit cellular activation during LH surge, and are considered essential for GnRH-induced preovulatory LH surge (Kauffman et al., 2014; McQuillan et al., 2019; Poling et al., 2017; Smith et al., 2006; Williams et al., 2011; Yip et al., 2015). On the other hand, *Kiss1* mRNA expression is downregulated by E2 in the ARH (Smith et al. 2005a, 2005b). ARH kisspeptin cells project to GnRH neurons' distal terminals near the median eminence instead of the soma (Yip et al., 2015; Yip et al., 2021) and are considered the pulse generator responsible for releasing GnRH and controlling the pulsatile LH secretion (d'Anglemont de Tassigny et al., 2008; Lehman et al., 2018; Yeo and Herbison 2014). The preoptic area, containing GnRH and kisspeptin cells, and the ARH are among the hypothalamic regions that express VIP receptors (VPACR) (Campbell et al., 2017; Kallo et al., 2004; Smith et al., 2000; Stephens and Kauffman 2021).

Exogenous VIP administration is able to stimulate GnRH neurons in an estradiol- and time of day-dependent manner in ovariectomized (OVX) females, or in a time of day-independent manner in gonad-intact mice, suggesting that this neurocircuit regulates the hypothalamic-pituitary-gonadal (HPG) axis (Christian and Moenter 2008; Piet et al., 2016; Samson et al., 1981). It has been proposed that this neuropeptide may provide an excitatory circadian signal to GnRH cells (van der Beek, 1994). However, inhibitory VIP-mediated effects on the HPG axis have also been described (Akema et al., 1988; Alexander et al., 1985; Kauffman et al., 2014; Sun et al., 2012; Weick and Stobie 1992). For example, intracerebroventricular (icv) infusion of VIP leads to a reduced percentage of GnRH and AVPV/PeN kisspeptin neurons coexpressing *C-fos* in young OVX rats as well as to decreased LH pulse frequency and mean LH levels (Alexander et al., 1985; Kauffman et al., 2014; Sun et al., 2012; Weick and Stobie 1992). Moreover, microinjection of VIP into the medial preoptic area attenuated LH secretion in OVX animals (Akema et al., 1988). Furthermore, it was reported that VIP induces a significant increase of the intracellular calcium levels ($[Ca^{2+}]_i$) in a subpopulation of ARH kisspeptin neurons in gonad-intact female mice (Schafer et al., 2018).

Despite the previous studies, the knowledge of the precise pathways and mechanisms governing the temporal control of the HPG axis remains incomplete. Thus, the present study investigated the potential effects of VIP on AVPV/PeN and ARH kisspeptin neurons in female mice during estrogen negative feedback, which is a condition particularly less studied regarding the physiological role of VIP on HPG axis.

2. Materials and methods

2.1. Animals

Kiss1-hrGFP (Stock No: 023425, The Jackson Laboratory, Bar Harbor, ME, USA), Kiss1-Cre/GFP (Cravo et al., 2011) and C57BL6 female mice were used in this study. At three weeks of age, Kiss1-hrGFP and Kiss1-Cre/GFP mice were weaned and genotyped via PCR with the DNA extracted from tail tips using the REDExtract-N-Amp Tissue PCR Kit (Sigma, St. Louis, USA). The mice were housed 4 to 5 animals per cage and kept in environmentally controlled rooms with a 12 h on/12 h off light cycle (lights on at 06:00 h) and a temperature between 21−23 °C. All animal procedures were approved by the Institute of Biomedical Sciences Animals Ethics Committee at the University of São Paulo and were performed according to the ethical guidelines adopted by the Brazilian College of Animal Experimentation.

2.2. Electrophysiological recordings

We performed whole-cell patch-clamp recordings on samples from 8 to 12 week old diestrus-stage or ovariectomized (OVX) Kiss1-hrGFP mice to examine the acute effects of VIP on the membrane excitability of kisspeptin neurons. Females with regular estrous cyclicity (i.e., diestrus), as evidenced by the daily observation of vaginal smears, were selected for electrophysiological experiments. The OVX females were obtained by performing a bilateral ovariectomy 7–10 days before tissue collection, as previously described (da Silva et al., 2014).

Mice were euthanized by decapitation (10:00 a.m.–11:00 a.m., circadian time (CT): 4–5), and then the entire brain was removed and immediately submerged in ice-cold, carbogen-saturated (95% O_2 and 5% CO_2) artificial cerebral spinal fluid (ACSF) composed of 238 mM sucrose, 2.5 mM KCl, 26 mM $NaHCO_3$, 1.0 mM NaH_2PO_4, 10 mM glucose, 1.0 mM $CaCl_2$ and 5.0 mM $MgCL_2$. Coronal sections from a hypothalamic block (250 μm) were cut with a Leica VT1000S vibratome (Leica Biosystems, Buffalo Grove, IL. USA; RRID: SCR_016495) and then incubated in oxygenated ACSF containing 124 mM NaCl, 2.8 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 5 mM glucose and 2.5 mM $CaCl_2$ at 30 °C for at least 1 h before the experiment. The slices were transferred to the recording chamber and allowed to equilibrate for 10–20 min before recording. The slices were bathed in oxygenated ACSF (30 °C) at a ~2 mL/min flow rate. The pipette solution for whole-cell recording contained 120 mM K-gluconate, 10 mM KCl, 10 mM HEPES, 5 mM EGTA, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 2 mM (Mg)-ATP, pH 7.3. Epifluorescence was briefly employed to target the fluorescent cells. The light source was then switched to infrared differential interference contrast imaging to obtain the whole-cell recording. A Leica DM6000 FS research system microscope equipped with a fixed stage and a Leica DFC360 FX high-speed monochrome fluorescence digital camera was used for data collection. The electrophysiological signals from AVPV/PeN and ARH kisspeptin neurons were recorded using an Axopatch 700B amplifier (Molecular Devices, San Jose, CA, USA; RRID: SCR_011323), low-pass filtered at 2–4 kHz, and analyzed offline on a PC using the pCLAMP program (Molecular Devices). The recording electrodes had resistances of 5–7 MΩ when filled internally with the K-gluconate solution. Data analysis was performed as previously described (Silveira et al., 2017). The input resistance was assessed by measuring the voltage deflection at the end of the response to a hyperpolarizing rectangular current pulse (500 ms of −10 to −30 pA). The membrane potential values were compensated to account for the liquid junction potential (−8 mV). The solution containing VIP (1 μM) was perfused for approximately 5 min. Some recordings were performed with ACSF containing tetrodotoxin (TTX, 1 μM) or the amino acid receptor antagonists: 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM), 2-amino-5-phosphonovalerate (AP-5, 50 μM) or picrotoxin (50 μM) to determine whether VIP effects depend on action potentials (APs) mediated by synaptic transmission. The VIP concentration used was based on previous studies which have demonstrated that these concentrations are effective in activating firing rate or changing $[Ca^{2+}]_i$ of kisspeptin or GnRH neurons (Piet et al., 2016; Schafer et al., 2018). TTX and the amino acid receptor antagonists concentrations were based on previous studies which have demonstrated the ability to block Na^+ conductance (TTX), ionotropic glutamatergic receptors (CNQX, AP5), and $GABA_A$ receptors (picrotoxin) (Berg et al., 2018; Frazao et al., 2013; Piet et al., 2016). TTX was obtained from Alomone Labs; CNQX, AP-5, picrotoxin and VIP were purchased from Tocris Bioscience. The AP frequency (*f*Aps) was determined by comparing the average firing rates 2 min immediately before and 2 min while applying the drugs in the bath. The *f*Aps were analyzed with the Mini Analysis Program (Synaptosoft, Decatur, GA, USA; RRID: SCR_002184).

2.3. Relative gene expression

Female C57BL6 mice (10–12 weeks of age) were distributed into

three experimental groups, diestrus (control), OVX, and OVX + E2. For the diestrus group, tissue collection was performed on the second day of diestrus (n = 6). OVX mice were submitted to bilateral ovariectomy under isofluorane anesthesia ten days before tissue collection (n = 5). OVX + E2 mice were submitted to bilateral ovariectomy and simultaneously implanted with a SILASTIC capsule (Dow- Corning, inner diameter, 1.98 mm; outer diameter, 3.18 mm) containing 1 μg of 17β-estradiol (Sigma) suspended in sesame oil (n = 7) three days before tissue collection. This dose of E2 has been previously shown in mice to elevate serum E2 levels, increase uterine mass, regulate *Kiss1* expression in the hypothalamus and properly suppress LH levels (Frazao et al., 2013). Importantly, the expected E2 levels are considered physiological (Nelson et al., 1992). Of note, in current study, OVX + E2 mice exhibited increased uterine mass (0.186 ± 0.02 g, n = 6) compared to the OVX animals (0.016 ± 0.001 g, n = 5; $P = 0.0002$).

The mice were anesthetized, euthanized by decapitation, and the entire brain was removed between 10:00 a.m.-12:00 p.m. (CT4- CT6). Brain coronal sections (250 μm) were cut with a vibratome. A single midline micro-punch of the AVPV/PeN (bregma 0.38 to 0.02 mm) was obtained from a coronal section using an 18-gauge needle. We used the mouse brain atlas as an anatomical reference (Franklin and Paxinos 2008). Two additional coronal sections of 500 μm were cut (relative to bregma −1.22 to −2.18 mm), and bilateral punches to collect the ARH were performed using an 18-gauge needle.

Total RNA from the micropunches was extracted with the PicoPure RNA isolation kit (Thermo Fisher Scientific Cat# KIT0204, Waltham, MA USA; RID: SCR_008817), according to the instructions of the manufacturer. An Epoch Microplate Spectrophotometer (Gen5, BioTek, Winooski, VT, USA; RRID: SCR_017317) was used to determine RNA quantity (Bohlen et al., 2019). Reverse transcription was performed by combining 0.5 μg of total RNA from the AVPV/PeN punches, or 1.0 μg of total RNA from ARH punches, with the SuperScript II Reverse Transcriptase (Thermo Fisher Scientific) and random primers p(dN)6 (Roche Applied Science). Real-time polymerase chain reaction (RT-PCR) was performed using a 7500 Real-Time PCR System (Thermo Fisher Scientific) and the Power SYBR Green PCR Master Mix (Thermo Fisher Scientific). All samples were run in duplicate, and negative controls were included on each qPCR plate. Relative mRNA expression was quantified by calculating $2^{-\Delta\Delta}$Ct. Data were normalized to the geometric average of the housekeeping genes *Actb*, *Gapdh*, *Ppia* and were reported as fold change compared to values obtained from the control group (set to 1.0). Importantly, the relative to mean of *Actb*, *Gapdh*, and *Ppia* was not different among the groups in the punches obtained from the AVPV/PeN or ARH (data not shown). The following primers (presented from the 5′ to 3′ ends) were used for the RT-PCR analysis: *Actb* (forward: gctccggcatgtgcaaag; reverse: catcacaccctggtgccta), *Gapdh* (forward: gggtcccagcttaggttcat; reverse: tacggccaaatccgttcaca), *Kiss1* (forward: gattccttttcccaggcatt; reverse: ggcaaaagtgaagcctggat), *Ppia* (forward: cttcttgctggtcttgccattcc reverse: tatctgcactgccaagactgagt), *Tac2* (forward: ccgctccatctctctggaag; reverse: tgcatgtcacgtttctgtgg), *Vpac1* (forward: cggccacccgacattgggaag; reverse: ctgcatgtggcgccgttgctg), *Vpac2* (forward: aatgaccagtcacagtacaaga; reverse: tcacactgtacctcactgttca).

2.4. Double immunofluorescence

The icv cannulas (Plastics One) were implanted into the lateral ventricle of adult Kiss1-Cre/GFP female mice, after which they were allowed to recover for approximately five days. Mice on the first day of diestrus received an acute icv injection of saline (2 μl) or VIP (1 μM) 1 h after lights on (CT1), and 15 min later mice were anesthetized with isoflurane and perfused transcardially with saline, followed by a 10% buffered formalin solution. The brains were collected and post-fixed in the same fixative for 120 min and cryoprotected in 0.1 M PBS containing 20% sucrose overnight at 4 °C. Then, the brains were cut into 30-μm thick sections using a freezing microtome. Brain slices were rinsed in 0.02 M potassium PBS (KPBS), pH 7.4, followed by incubation in 3% normal serum for 1 h. The sections were incubated overnight with anti-GFP (1:5000; Aves Labs, Inc.; #GFP-1020) and anti-phospho^{Ser133} cAMP-response-element-binding protein (pCREB; 1:1000; Cell Signaling Technology; #9198) antibodies. Then the sections were incubated with Alexa Fluor-conjugated secondary antibodies (1:500; Jackson ImmunoResearch Laboratories) for 90 min. After rinsing the sections with KPBS, they were mounted onto gelatin-coated slides and covered with Fluoromount G mounting medium. Correct placement of the cannula into the lateral ventricle was confirmed by histological inspection. A Zeiss Axiocam 512 color camera adapted to an Axioimager A1 microscope (Zeiss, Munich, Germany) was used to obtain the photomicrographs.

2.5. Statistical analysis

The data presented herein are expressed as the mean ± standard error of the mean (SEM). We used ANOVA and Tukey's post-test to compare the gene expression data. Repeated Measures ANOVA and Tukey's post-test were used to evaluate electrophysiological data. The unpaired two-tailed Student's t-test was used for making comparisons between two groups. Fisher's exact test was used for comparisons in the percentage of responsive cells. Results with P values of <0.05 were considered statistically significant. Statistical analyses were performed using the GraphPad Prism software (GraphPad Prism, San Diego, CA, USA; RRID: SCR_002798).

3. Results

3.1. VIP depolarizes a subset of AVPV/PeN kisspeptin neurons

We evaluated the effects of VIP in the resting membrane potential (RMP) of kisspeptin neurons using whole-cell patch-clamp in current-clamp mode, under zero current injection (I = 0). We identified kisspeptin neurons by the hrGFP reporter protein, whose expression is transcriptionally regulated by *Kiss1* gene, as previously demonstrated (Cravo et al., 2013). We recorded AVPV/PeN kisspeptin neurons from gonad-intact female mice (diestrus-stage). Kisspeptin neurons (17 cells from 13 animals) exhibited an average RMP of −64.4 ± 2.6 mV (range: -84 to - 48 mV) and input resistance of 1.0 ± 0.1 GΩ. Among the recorded neurons, 7 out of 17 AVPV/PeN kisspeptin cells showed spontaneous discharges of APs at rest (1.4 ± 0.3 Hz). VIP induced depolarization in the RMP of 5 out of 17 (30%) neurons recorded in the AVPV/PeN (Fig 1A, C-E). The depolarization was followed by a slight, non-significant increase of the input resistance and *f*APs (Fig. 1F and G). The remaining AVPV/PeN kisspeptin neurons were unresponsive to VIP administration. VIP induced no effects in RMP (−65.1 ± 3.4 mV), input resistance (0.9 ± 0.1 GΩ) or *f*APs (1.3 ± 0.3 Hz) in 12 out of 17 neurons (baseline: RMP: −65.0 ± 3.5 mV; IR: 0.9 ± 0.1 GΩ; *f*APs: 1.02 ± 0.3 Hz; $P > 0.05$).

Next, we determined whether the VIP-induced depolarization depends on AP-mediated synaptic transmission. Thus, brain slices were pretreated with TTX and amino acid receptor antagonists. Under such conditions, AVPV/PeN kisspeptin neurons (12 cells from 6 animals) exhibited RMP of – 60.4 ± 2.7 mV (range: - 84 to −50 mV), and the average input resistance was 1.3 ± 0.2 GΩ. In 33% (4 out of 12 cells) of recorded AVPV/PeN kisspeptin neurons, VIP still induced a depolarization in the RMP (Fig 1B, H–K). The remaining AVPV/PeN kisspeptin neurons (8 out of 12 cells) were unresponsive to VIP in the presence of TTX and amino acid receptor antagonists (baseline: RMP: −59.7 ± 3.8 mV; IR: 1.4 ± 0.2 GΩ; VIP: −59.8 ± 3.8 mV; IR: 1.4 ± 0.3 GΩ; washout: RMP: −60.2 ± 4.3 mV; IR: 1.3 ± 0.3 GΩ; $P > 0.05$). Importantly, the percentage of responsive cells was similar between the recordings performed with and without TTX and amino acid receptor antagonists ($P > 0.05$). Taken together, approximately one third of AVPV/PeN kisspeptin neurons are directly excited by VIP.

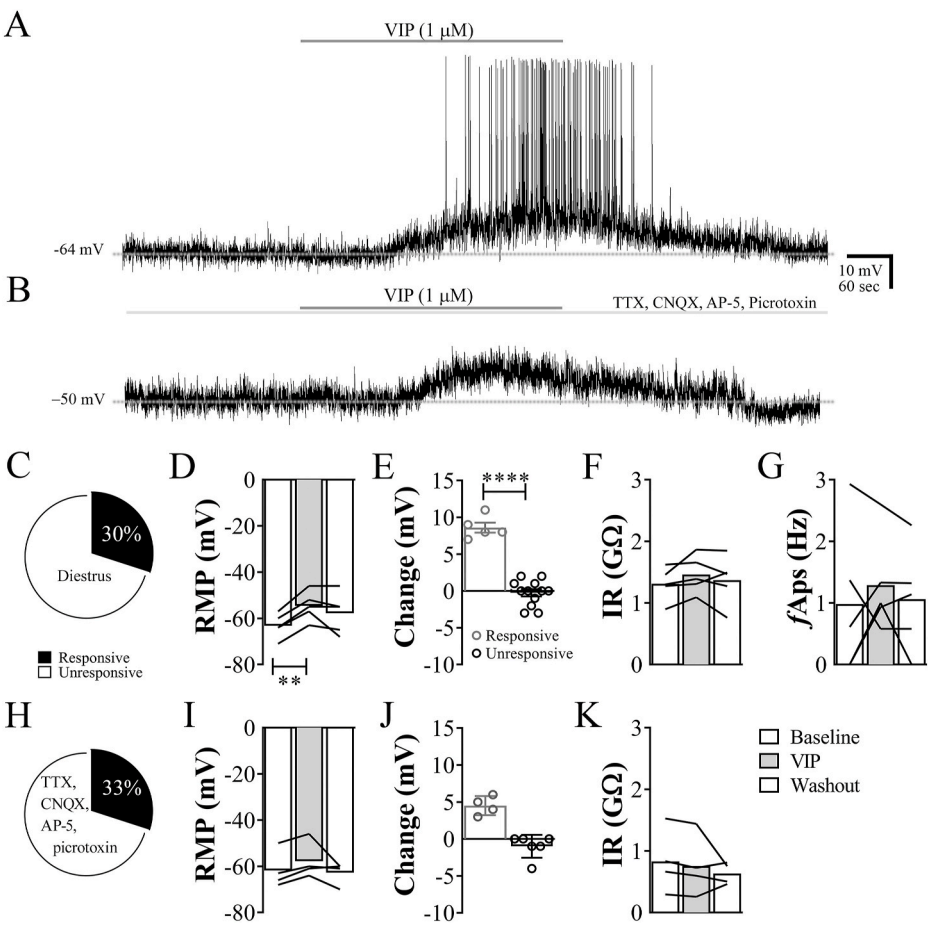

Fig. 1. VIP induces the depolarization of a subset of kisspeptin neurons located in AVPV/PeN. (A) Representative current-clamp recording demonstrating VIP-induced depolarization of AVPV/PeN kisspeptin neurons. (B) Representative current-clamp recording demonstrating VIP-induced depolarization in ACSF containing tetrodotoxin (TTX) and the amino acid receptor antagonists (6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 2-amino-5-phosphonovalerate (AP-5) and picrotoxin). The dashed line indicates the resting membrane potential (RMP). (C, H) Pie chart showing the percentage of VIP responsive cells (5 out of 17 cells from 13 animals) recorded from females in diestrus in regular ACSF (C) or ACSF containing tetrodotoxin (TTX) and the amino acid receptor antagonists (4 out of 12 cells from 6 animals; H). Bar graphs demonstrating the average RMP (D, I), change in RMP (E, J), input resistance (IR, F, K) and frequency of action potentials (*f*Aps, G) of VIP responsive cells recorded in regular ACSF (D–G) or ACSF containing tetrodotoxin (TTX) and the amino acid receptor antagonists (I–K). $^{**}P < 0.006$, $^{****}P < 0.0001$.

3.2. VIP depolarizes ARH kisspeptin neurons

Next, we assessed the effects of VIP on the RMP of ARH kisspeptin neurons by recording cells from female mice (diestrus-stage). The average RMP of ARH kisspeptin neurons (14 cells from 11 animals) was - 56.1 ± 1.3 mV (range: - 67 to - 49 mV), and the average input resistance was 1.3 ± 0.1 GΩ. Most ARH kisspeptin neurons were quiescent at rest, whereas 6 out of 14 (approximately 42%) cells showed spontaneous APs with *f*APs of 1.5 ± 1.1 Hz. VIP induced depolarization in 93% (13 out of 14 recorded cells) of ARH kisspeptin neurons (Fig. 2A, C-E). Moreover, VIP-mediated depolarization induced a significant increase in the IR and *f*APs (Fig. 2F and G). After washout for up to 20 min, the effects of VIP were not completely reversed.

The effects of VIP on ARH kisspeptin neurons were further evaluated in the presence of TTX and amino acid receptor antagonists. Under these conditions, ARH kisspeptin neurons exhibited a RMP of - 53.9 ± 2.1 mV (range: - 77 to - 40 mV) and an average IR of 1.0 ± 0.1 GΩ (19 cells from 8 animals). VIP-induced depolarization was also observed in 84% (16 out of 19 cells) of ARH kisspeptin neurons co-treated with TTX and amino acid receptor antagonists (Fig 2B, H-J). IR remained unchanged (Fig. 2K). The remaining ARH kisspeptin neurons (3 out of 19 cells) were unresponsive to VIP administration in the presence of TTX and amino acid receptor antagonists (Baseline: RMP: −46.3 ± 3.8 mV; IR: 1.0 ± 0.3 GΩ; VIP: RMP: −45.6 ± 4.2 mV; IR: 0.9 ± 0.2 GΩ; washout: RMP: −47.0 ± 4.2 mV; IR: 1.0 ± 0.2 GΩ; $P > 0.05$). The percentage of ARH responsive cells was not different between the recordings performed with and without TTX and amino acid receptor antagonists ($P > 0.05$). These results demonstrate that VIP directly excited the majority of ARH kisspeptin neurons.

3.3. Icv VIP injection induces pCREB in AVPV/PeN kisspeptin neurons but not in ARH kisspeptin neurons

In addition to the effects of VIP on the RMP of kisspeptin neurons, VIP may also regulate other cellular aspects, including the activation of intracellular signaling pathways. Belonging to the GαS protein-coupled receptor family, VIP receptors activate the adenylyl cyclase enzyme, increasing cyclic adenosine monophosphate (cAMP) levels (Langer 2012). The subsequent increase in intracellular cAMP concentration leads to protein kinase A activation and the phosphorylation of downstream targets, such as CREB at the Ser133 residue. pCREB acts as a critical transcription factor that controls the expression of several immediate early genes (Naqvi et al., 2014). Thus, we determined whether an acute icv injection of VIP could shift the percentage of kisspeptin neurons containing pCREB. We observed that icv VIP injection increased in 40% the percentage of kisspeptin neurons containing pCREB in the AVPV/PeN of female mice compared to saline-injected mice (Fig. 3A–C). In contrast, no significant differences in the percentage of kisspeptin neurons expressing pCREB in the ARH were observed between VIP- and saline-injected mice (Fig. 3A–B, D).

3.4. Vpac1 and Vpac2 mRNA levels in the AVPV/PeN are modulated by serum estradiol levels

Previous studies have indicated that VIP-mediated effects on the HPG axis may be estradiol-dependent (Christian and Moenter 2008). We determined the influence of serum estradiol levels on *Vpac1* and *Vpac2* mRNA levels in the AVPV/PeN and ARH. *Kiss1* and *Tac2* mRNA levels were considered control transcripts since estradiol-induced changes in the expression of these genes have been well-described (Smith et al.,

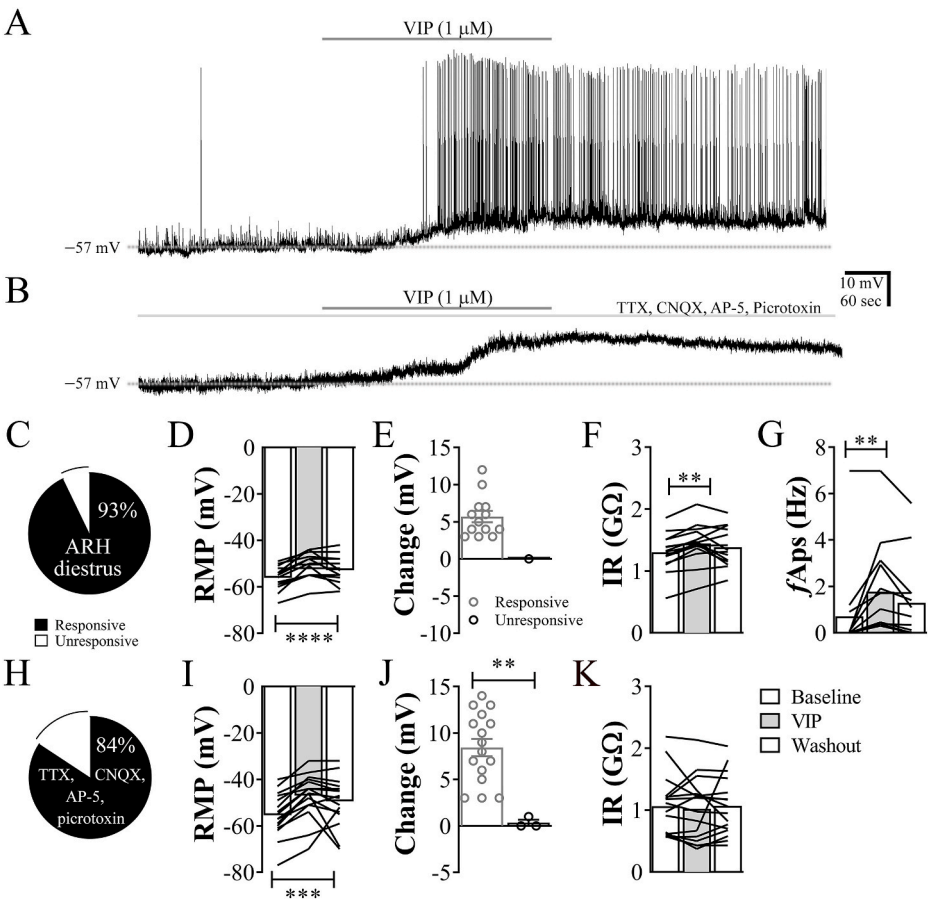

Fig. 2. VIP depolarizes most kisspeptin neurons located at the ARH. (A) Representative current-clamp recording demonstrating the VIP-induced depolarization of ARH kisspeptin neurons. (B) Representative current-clamp recording demonstrating VIP-induced depolarization in ACSF containing tetrodotoxin (TTX) and the amino acid receptor antagonists (6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 2-amino-5-phosphonovalerate (AP-5) and picrotoxin). The dashed line indicates the resting membrane potential (RMP). (C, H) Pie chart showing the percentage of VIP responsive cells (13 out of 14 cells from 11 animals) recorded from females in diestrus in regular ACSF (C) or ACSF containing tetrodotoxin (TTX) and the amino acid receptor antagonists (16 out of 19 cells from 8 animals; H). Bar graphs demonstrating the average RMP (D, I), change in RMP (E, J), the input resistance (IR, F, K) and the frequency of action potentials (*f*Aps, G) of VIP responsive cells recorded in regular ACSF (D–G) or ACSF containing tetrodotoxin (TTX) and the amino acid receptor antagonists (I–K). $^{**}P < 0.006$, $^{***}P \leq 0.0002$, $^{****}P < 0.0001$.

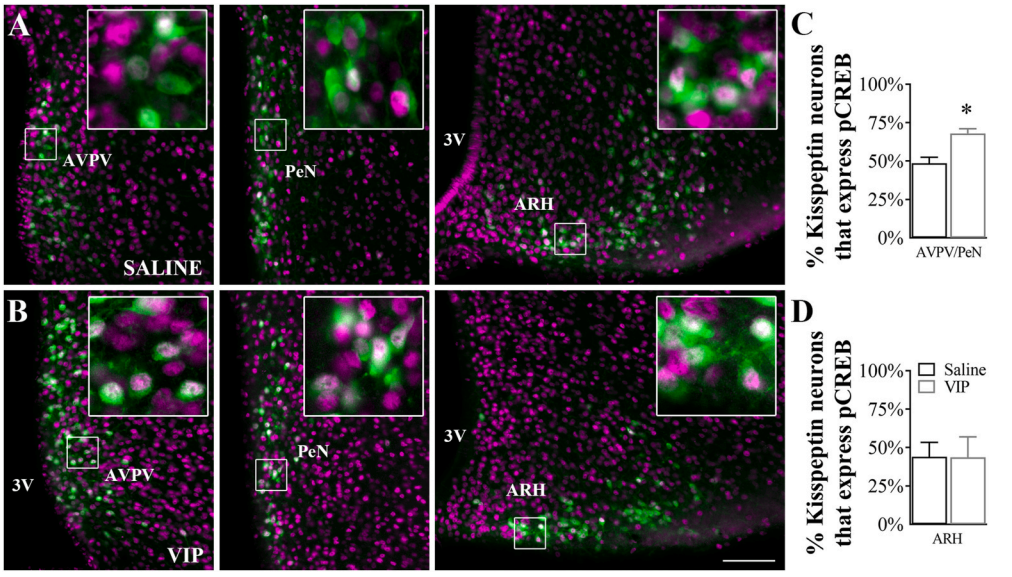

Fig. 3. Intracerebroventricular VIP injection increases pCREB levels in AVPV/PeN kisspeptin neurons but not in ARH kisspeptin neurons. (A–B) Brain sections of Kiss1-Cre/GFP female mice on the first day of diestrus-stage that received an intracerebroventricular injection of saline (A) or VIP (B) and were perfused 15 min later. Green staining shows GFP (kisspeptin) neurons, and the nuclear magenta staining shows $pCREB^{Ser133}$. The insets represent higher magnification images. C-D. Percentage of kisspeptin neurons that co-express pCREB in saline-injected (n = 5) and VIP-injected (n = 4) mice. Abbreviation: 3V, third ventricle. Scale bar = 100 μm $^{*}P$ = 0.0073. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2005a). As expected, sex steroid levels influenced *Kiss1* ($F_{(2,\ 29)} = 9.859$; $P = 0.0005$; Fig. 4A) mRNA levels in the AVPV/PeN and ARH, and *Tac2* expression ($F_{(2,\ 30)} = 9.602$; $P = 0.0006$; Fig. 4B) in the ARH. Under absent sex steroid levels, *Kiss1* mRNA was downregulated in the AVPV/PeN, whereas *Kiss1* and *Tac2* mRNA levels were upregulated in the ARH (Fig. 4A and B). Furthermore, a significant interaction between the hypothalamic nuclei and sex-steroid levels were observed with *Kiss1* ($F_{(2,\ 29)} = 16.26$; $P < 0.0001$; Fig. 4A) and *Tac2* ($F_{(2,\ 30)} = 11.24$; $P = 0.0002$; Fig. 4B) expression.

A significant sex steroid-dependent effect was also observed with *Vpac1* ($F_{(2,\ 28)} = 3.561$; $P = 0.0419$; Fig. 4C) and *Vpac2* ($F_{(2,\ 26)} = 3.646$; $P = 0.0402$; Fig. 4D) mRNA levels. Post hoc analysis evaluating the effects within each nucleus demonstrated that *Vpac1* mRNA levels were upregulated in the AVPV/PeN of OVX mice (diestrus vs OVX, $P = 0.0007$; diestrus vs OVX + E2, $P = 0.4774$, OVX vs OVX + E2, $P = 0.0092$). A similar tendency was observed with *Vpac2* mRNA expression

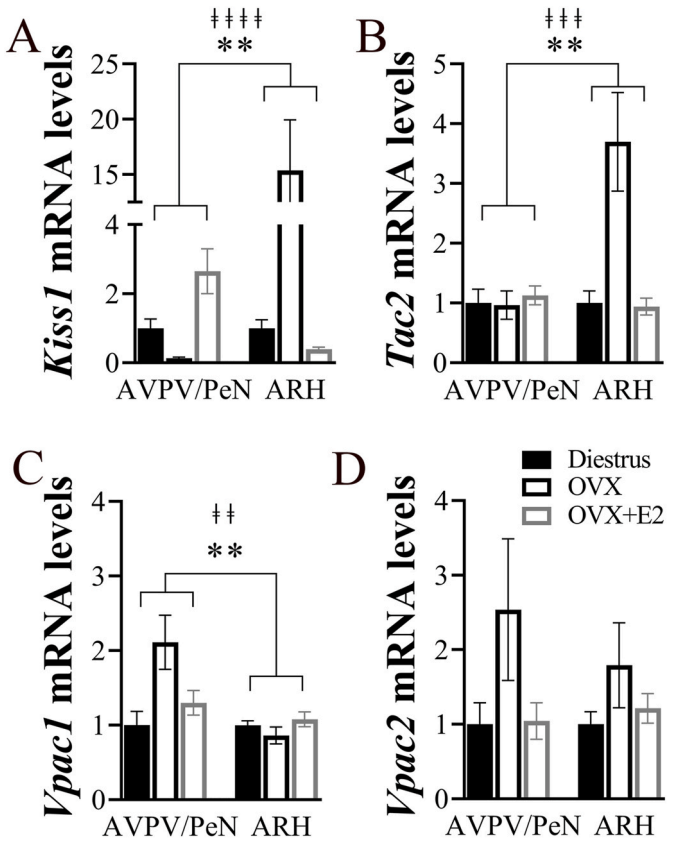

Fig. 4. Absent sex steroids levels (OVX) lead to the upregulation of genes coding for VPACR in the AVPV/PeN. (A–D) Bar graphs comparing *Kiss1* (A), *Tac2* (B), *Vpac1* (C) and *Vpac2* (D) mRNA expression in the AVPV/PeN and arcuate nucleus of the hypothalamus (ARH). The AVPV/PeN or ARH punches were obtained from diestrus-stage (n = 5/6), ovariectomized (OVX, n = 5) and OVX estradiol (E2)-primed mice (OVX + E2, n = 6/7). * Indicates significant difference between brain nuclei, $^{**}P < 0.005$. ǂ indicates significant interaction between the hypothalamic nuclei and sex-steroid levels, $^{ǂǂ}P < 0.005$, $^{ǂǂǂ}P < 0.0005$, $^{ǂǂǂǂ}P < 0.0001$.

levels; however, the extent of this increase failed to reach a level of statistical significance (diestrus vs OVX, $P = 0.0773$; diestrus vs OVX + E2, $P = 0.9975$, OVX vs OVX + E2, $P = 0.0722$). The upregulation of genes encoding for VPACR was specific to the AVPV/PeN, since no differences in *Vpac1* (diestrus vs OVX, $P = 0.8513$; diestrus vs OVX + E2, $P = 0.9398$, OVX vs OVX + E2, $P = 0.6573$) or *Vpac2* (diestrus vs OVX, $P = 0.4803$; diestrus vs OVX + E2, $P = 0.9415$, OVX vs OVX + E2, $P = 0.6497$) mRNA levels were detected in the ARH. Further comparisons of gene expression levels in the AVPV/PeN and ARH revealed higher *Vpac1* mRNA expression in the AVPV/PeN compared to ARH ($F_{(1, 28)} = 11.40$; $P = 0.0022$; Fig. 4C), whereas *Vpac2* mRNA levels were similar between these hypothalamic nuclei ($F_{(1, 26)} = 0.2560$; $P = 0.6171$; Fig. 4D). A significant interaction between the hypothalamic nuclei and sex-steroid levels was observed with *Vpac1* expression ($F_{(2, 28)} = 6.594$; $P = 0.0045$; Fig. 4C), but not that of *Vpac2* ($F_{(2, 26)} = 0.5474$; $P = 0.5850$; Fig. 4D). Thus, the expression of VIP receptors is upregulated in the AVPV/PeN in OVX mice.

3.5. Changes in sex steroid levels do not affect AVPV/PeN kisspeptin neuron VIP responsiveness

Since the expression of VIP receptors in the AVPV/PeN is upregulated in situations of absent sex steroid levels, we next recorded the AVPV/PeN kisspeptin neurons from OVX mice. The RMP and IR were - 59.7 ± 4.1 mV (range: - 80 to - 43 mV) and 1.2 ± 0.1 GΩ, respectively (10 cells from 5 animals). We observed no significant differences comparing the RMP and IR values between diestrus and OVX mice ($P > 0.05$). Among the recorded neurons, 5 out of 10 AVPV/PeN kisspeptin cells showed spontaneous APs at rest (*f*APs: 1.4 ± 0.6 Hz). VIP depolarized 40% (4 out of 10 cells) of the AVPV/PeN kisspeptin neurons in OVX mice (Fig. 5A–D). The depolarization of RMP was followed by a slight non-significant increase of the IR, but no changes in the *f*APs were observed (Fig. 5E and F). The remaining AVPV/PeN kisspeptin neurons (6 out of 10) were unresponsive to VIP administration to the bath (baseline: RMP: −58.8 ± 6.9 mV; IR: 1.1 ± 0.2 GΩ; *f*APs: 1.1 ± 0.6 Hz; VIP: RMP: −58.8 ± 6.5 mV; IR: 1.1 ± 0.2 GΩ; *f*APs: 1.1 ± 0.7 Hz; $P > 0.05$). Of note, the percentage of responsive cells was similar between diestrus and OVX mice ($P > 0.05$). These findings suggest that VIP responsiveness in AVPV/PeN kisspeptin neurons is relatively similar between gonad-intact and OVX mice.

4. Discussion

In the present study, we demonstrated that VIP directly affects kisspeptin neurons during estrogen negative feedback in diestrus females through at least two mechanisms. The first mechanism is related to VIP-induced modulation of the membrane potential of AVPV/PeN and ARH kisspeptin neurons. The second mechanism involves VIP-induced pCREB signaling only in AVPV/PeN kisspeptin cells. Interestingly, we found that VPACR expression in the brain area containing the AVPV/PeN, but not in ARH punches, is modulated by estradiol levels. Despite the upregulated VPACR gene expression in OVX mice, the percentage of VIP-responsive AVPV/PeN cells was similar between gonad-intact and OVX mice. Therefore, the potent activation of hypothalamic kisspeptin neurons suggests that VIP modulate the HPG axis during estrogen negative feedback.

Kauffman et al. (2014) reported that VIP partially prevents the induction of *C-fos* expression in kisspeptin neurons at the time of LH surge in OVX + E2 young rats, but not in middle-aged females. Despite the evident ability of the VIP/VPACR interaction to mediate a pathway that leads to *C-fos* expression, few studies have described the action of VIP in kisspeptin neurons (Kauffman et al., 2014; Schafer et al., 2018). Herein, we demonstrated that VIP depolarizes the RMP and induces CREB phosphorylation in a subset of AVPV/PeN kisspeptin cells during estrogen negative feedback. Since we analyzed VIP effects in mice on diestrus or after OVX, our recordings were focused predominantly in AVPV/PeN neurons that express *Kiss1* under estrogen negative feedback. Thus, a different percentage of responsiveness to VIP could be observed if AVPV/PeN kisspeptin neurons that mediate the positive estradiol feedback were also recorded. Considering that our goal was to understand whether VIP mediates kisspeptin activity under estrogen negative feedback, an excitatory effect on the RMP of AVPV/PeN kisspeptin cells in diestrus-stage or in OVX mice may appear contradictory. Other counterintuitive effects in AVPV/PeN kisspeptin neurons activity have been observed when studying prolactin or vasopressin effects in this population (Silveira et al., 2017; Williams et al., 2011). It has been shown that kisspeptin neurons mediate the inhibitory effect of prolactin on the HPG axis (Ribeiro et al., 2015; Szawka et al., 2010). Prolactin suppresses hypothalamic expression of *Kiss1* mRNA and LH plasma levels during estrogen negative feedback, even though a small subset of AVPV/PeN kisspeptin neurons are excited by this hormone (Araujo-Lopes et al., 2014; Silveira et al., 2017). On the other hand, vasopressin injections can initiate the LH surge in OVX + E2 rats only during the afternoon (Palm et al., 2001). Even though, vasopressin modulates AVPV/PeN kisspeptin activity, using Fos expression as a marker, either in the morning or in the afternoon, while GnRH neurons exhibit time-dependent responsivenesses to vasopressin (Palm et al., 2001; Williams et al., 2011).

As mentioned by Stephens and Kauffman (2021), our knowledge of the various functions of AVPV kisspeptin cells seems to be limited. However, there is evidence that AVPV/PeN kisspeptin neurons are actually a heterogeneous group of cells. For example, Kumar et al.

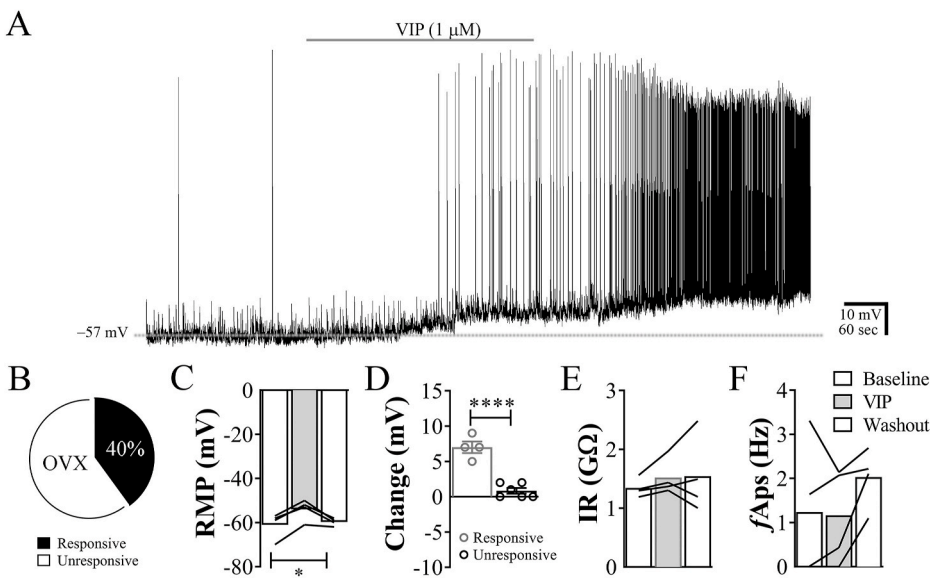

Fig. 5. VIP responsiveness in AVPV/PeN kisspeptin neurons is similar in diestrus and OVX mice. (A) Representative current-clamp recording demonstrating VIP-induced depolarization of an AVPV/PeN kisspeptin neurons recorded from ovariectomized (OVX) mice. The dashed line indicates the resting membrane potential (RMP). (C) Pie chart showing the percentage of VIP responsive cells (4 out of 10 cell from 5 animals) recorded from OVX mice in regular ACSF. Bar graphs demonstrating the average RMP (C), change in RMP (D), the input resistance (E) and the frequency of action potentials (*f*Aps, F) of VIP responsive cells recorded in regular ACSF. $^{*}P < 0.05$, $^{****}P < 0.0001$.

(2015) has shown that only 36% of AVPV kisspeptin neurons communicate with GnRH neurons in adult female mice. Despite this "small percentage", it is well-known that AVPV/PeN kisspeptin neurons are major regulators of the activity of GnRH neurons to induce the pre-ovulatory LH surge (Lehman et al., 2018). Accordingly, approximately 40% of AVPV kisspeptin neurons express C-*fos* mRNA or Fos protein at the LH surge (Kauffman et al., 2014; Williams et al., 2011). It is possible that just a subset of AVPV kisspeptin neurons expresses VPACR or somehow is responsive to VIP stimulatory effect at the time of our recordings. We also showed that not only VIP affects the membrane potential of approximately 30% of AVPV/PeN cells during diestrus, but it also recruits CREB pathway in AVPV/Pen cells. Considering that AVPV/PeN kisspeptin neurons coexpress multiple genes, some of them are modulated by E_2 levels (Stephens and Kauffman 2021), the excitatory effect of VIP on AVPV/PeN kisspeptin cells under estrogen negative feedback may represent an intrinsic mechanism that contributes somehow to functions other than direct modulation of GnRH secretion. In addition to controlling the preovulatory LH surge, AVPV/PeN kisspeptin neurons modulate sexual behavior (Hellier et al., 2018). Moreover, reports suggest that VIP may stimulate prolactin secretion (Akema et al., 1988; Reichlin 1988) and can be involved in sexual behavior, at least in male rats and mice (Gozes et al., 1993; Gozes et al., 1989). Whether VIP-mediated effects in AVPV/PeN kisspeptin cells contribute to the modulation of PRL secretion or mouse sexual behavior deserves further investigation.

It has been previously demonstrated that VIP changes the $[Ca^{2+}]_i$ of approximately 60% of kisspeptin cells in the caudal ARH of female mice (Schafer et al., 2018), whereas only few kisspeptin cells were responsive to VIP in the caudal ARH of male mice. Furthermore, a very small percentage of kisspeptin cells in other ARH subregions was responsive to VIP (Schafer et al., 2018). These results suggest a sexually dimorphic and region-specific VIP effect on ARH kisspeptin neurons (Schafer et al., 2018). Here, we studied kisspeptin cells only in female mice; therefore, we cannot speculate whether VIP exerts a sexually dimorphic modulation in the membrane activity of kisspeptin neurons. It is important to point out that, in our study, most of the recorded ARH kisspeptin neurons were depolarized by VIP. Moreover, VIP-induced depolarization was TTX and amino acid receptor antagonists-insensitive and occurred independently of the rostrocaudal location of the recorded cell in the ARH (data not shown). Therefore, differences in the experimental approaches between our study and others may explain the differences in the responsiveness to VIP. Some groups have reported the ability of VIP to increase $[Ca^{2+}]_i$ levels without affecting the coupling to adenylyl cyclase (Dickson and Finlayson 2009). It has also been demonstrated that VPAC1R can interact with receptor activity-modifying proteins (RAMPs), particularly RAMP2, inducing a significant enhancement of agonist-mediated inositol trisphosphate production and the subsequent effect on cellular $[Ca^{2+}]_i$ levels (Christopoulos et al., 2003). Additionally, other second messengers which lead to modulation of $[Ca^{2+}]_i$ levels may also be induced upon VPACR stimulation (Dickson and Finlayson 2009). Thus, whether VIP-induced changes in the RMP of kisspeptin neurons depend on second messengers needs to be explored.

Numerous factors can regulate CREB phosphorylation which explains the large number of pCREB positive neurons labeled in the vehicle group. As a consequence, a high basal CREB phosphorylation in the hypothalamus is in accordance with previous studies from our group (Campos et al., 2021) and others (Perello et al., 2006). However, like others "unspecific" markers of neuronal activation (e.g., *C-fos*), pCREB has been frequently used as an indicator of acute stimuli. Importantly, the percentage of AVPV/PeN kisspeptin neurons expressing pCREB raised from 48% in vehicle group to 68% in the VIP-injected group, representing a ~40% increase, which is in accordance with the percentage of AVPV/PeN kisspeptin neurons that exhibited VIP-induced depolarization. Nonetheless, no significant effect on pCREB was observed in the ARH. Our results support that VIP modulates AVPV/PeN kisspeptin neurons by at least two signaling pathways during estrogen negative feedback in diestrus females. Considering that VIP can recruit different signaling pathways to modulate cell activity (Couvineau and Laburthe 2012; Dickson and Finlayson 2009; Harmar et al., 2012), it is plausible to think that alternative signaling pathways may promote the opening or the closure of plasma membrane ion channels, which explains the short-term effects in the electrical activity of target cells, instead of inducing CREB phosphorylation. Anyhow, we were not able to determine how pCREB modifies cellular functions in the AVPV/PeN compared to ARH kisspeptin neurons. VIP-induced CREB phosphorylation, and therefore the potential genomic effects, in AVPV/PeN kisspeptin cells needs to be further investigated. Furthermore, the physiological relevance of the VIP-mediated effects in kisspeptin cells through ionic membrane channels or via pCREB needs to be further clarified.

Pituitary adenylate cyclase-activating peptide (PACAP) shares 68% amino acid sequence homology with VIP (Dickson and Finlayson 2009; Miyata et al., 1989; Miyata et al., 1990) and also belongs to a structural family of related peptides referred to as the secretin receptor family or group B of the G-protein coupled receptor family. Both PACAP and VIP and their receptors, including VPACR, are spread throughout the body,

including the brain, and play diverse and essential roles in the central nervous system and peripheral tissues (Couvineau and Laburthe 2012; Harmar et al., 2012). Progress in dissociating the functions of VIP and PACAP is limited since VPAC1 and VPAC2 receptors do not discriminate between VIP and PACAP, and no selective drugs are available for determining affinity (Couvineau and Laburthe 2012; Harmar et al., 2012). Despite structural similarities, PACAP activation of VPACR does not seem to contribute to VIP-mediated effects on membrane activity (Ross et al., 2018). This conclusion is based on the fact that PACAP induces a region-dependent effect only on kisspeptin neuron $[Ca^{2+}]_i$ levels in the caudal extension of the ARH and does not affect rostral extension cells or AVPV/PeN kisspeptin neurons (Ross et al., 2018). Whether VPAC1R or VPAC2R differentially mediates VIP or PACAP effects in kisspeptin neurons also needs to be determined.

Previous studies postulated that reduced VIP secretion or reduced responsiveness to VIP might result in delayed or attenuated LH surge in middle-aged female rats (Kauffman et al., 2014; Sun et al., 2012). Furthermore, aging is characterized by reduced glutamatergic neurotransmission in the preoptic area, as well as the suppression of diurnal cAMP rhythms in the preoptic area and the SCN (Gerhold et al., 2005; Kiss et al., 2008; Neal-Perry et al., 2005; Neal-Perry et al., 2008). Even though OVX mice are a model for lack of sex steroid hormones, as occurs in menopause, we cannot extrapolate our results with this model to aging since these are entirely different processes. Nevertheless, attenuated VIP binding to its receptor could explain the upregulated VPACR gene expression in the AVPV/PeN of the OVX model, as suggested previously (Sun et al., 2012). Our study was not designed to determine whether increased VPACR mRNA levels in the OVX model were specific to kisspeptin neurons (isolate kisspeptin cells). Instead, we investigated *Vpac1* and *Vpac2* expression using tissue obtained in the entire nuclei which therefore includes other cells populations. Stephens and Kauffman (2021) recently demonstrated that AVPV kisspeptin neurons express *Vpac2*. *Vpac2* expression is not modulated by E2 levels in AVPV/PeN kisspeptin neurons (2021). In our study, we observed an upregulation of *Vpac2* mRNA levels in the AVPV/PeN punch. These results suggest that this effect is unspecific to kisspeptin neurons, especially when taking into account the fact that the responsiveness of AVPV/PeN kisspeptin neurons to VIP does not change in OVX mice. Whether kisspeptin neurons coexpress *Vpac1* needs to be further addressed.

Importantly, VIP-expressing neurons are located in multiple brain regions in addition to the SCN (Köhler 1982; Kosaka et al., 1985; Mezey and Kiss 1985; Rhomberg et al., 2018). Although it is known that LH surge is circadian in nature, only occurring at late afternoon/early evening in rodents, it was out of the scope of the current study to determine the source of VIP mediating estrogen negative feedback. Any brain area that contains VIP neurons and projects to AVPV/PeN or ARH nuclei can account for the observed effects.

5. Conclusion

Our findings contribute to the field demonstrating that VIP can modulate kisspeptin neurons activity during estrogen negative feedback in diestrus mice. Similar effects were also observed in OVX females lacking estrogen. Within the current knowledge is hard to draw a physiological correlation between the obtained results and the main function related to AVPV/PeN kisspeptin neurons. Differentiating whether AVPV/PeN neurons exhibit circadian time-dependent sensitivity to signals that initiate the LH surge, as previously demonstrated for GnRH neurons, can bring us some understanding and is an interesting field for further study (Williams et al., 2011; Zhao and Kriegsfeld 2009). The potent activation of ARH kisspeptin neurons suggests that VIP exerts an excitatory effect on the HPG axis and potentially contributes to pulsatile LH secretion during estrogen negative feedback.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Naira da Silva Mansano: Investigation, Data curation, Formal analysis, analyzed whole-cell patch-clamp data. **Regina Silva Paradela:** Investigation. **Tabata M. Bohlen:** Investigation. **Izabela M. Zanardi:** Investigation. **Fernanda Machado Chaves:** Investigation. **Marina Augusto Silveira:** Investigation, Data curation, Formal analysis, analyzed whole-cell patch-clamp data. **Mariana Rosolen Tavares:** Investigation. **Jose Donato:** Conceptualization, Writing – review & editing. **Renata Frazao:** Conceptualization, Data curation, Supervision, Writing – original draft.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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